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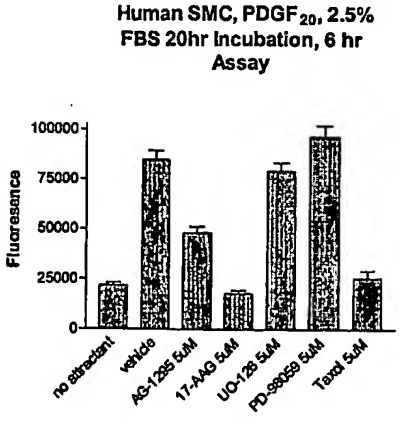
(54) Title: METHODS OF PREVENTING RESTENOSIS



(57) Abstract: The present invention is directed to a method of inhibiting smooth muscle cell migration both *in vitro* and *in vivo* by administering an effective amount of a compound that inhibits signaling in the Ras-Raf-Erk-MEKK1 pathway. The present invention is further directed to the treatment of restenosis by administering such a compound via an implantable stent. Examples of such compounds include benzoquinone ansamycins (e.g., 17-(allylamino)-17-demethoxygeldanamycin).

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METHODS OF PREVENTING RESTENOSIS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 60/506,116, filed September 26, 2003. The entire teachings of this provisional application are incorporated herein by reference.

BACKGROUND

Stents that are implanted in the wall of coronary arteries are often subject to restenosis or neointimal growth, whereby vascular smooth muscle cells (SMC), inflammatory cells, and extracellular matrix components, e.g., fibrin, contribute to reduce the lumen diameter of the vessel, thus requiring further treatment such as repeat procedures and coronary artery bypass surgery. It is well known that coating stents with certain drugs, formulated with or without polymers, can reduce the incidence of restenosis. Two drugs, paclitaxel (Taxol®) and rapamycin (Sirolimus®), are registered for use on drug eluting stents (DES), and other drugs that work via different pathways may also be useful on DES. Paclitaxel functions by modulating the structure of microtubules in cells, thus altering their ability to proliferate by blocking their progression through the cell cycle at mitosis. Rapamycin functions by modulating the intermediate signaling molecule, mTOR, resulting in reduced progression of cells from G_1 to S phases of the cell cycle.

The mitogenic signaling pathway that includes Ras, Raf, Erk1,2 and/or MEKK1 is required to transmit a variety of signals, e.g., from growth factors and cytokines such as PDGF or FGF. Blocking this pathway is known to reduce the proliferation and invasion of various human cell types (Kato-Stankiewicz et al (2002), PNAS 99:14398-14403), and by extension could also reduce the proliferation and invasion of SMC and other cell types involved in restenosis. Thus, inhibitors of this pathway that would be useful on DES include (but are not limited to): inhibitors of Ras or Raf; inhibitors of Ras-Raf interaction; inhibitors of Raf kinase; inhibitors of downstream signaling molecules such as Erk1,2 and MEKK1; and inhibitors of upstream activators of Ras or Raf such as PDGF receptor.

Compounds that inhibit signaling in the Ras-Raf-Erk-MEKK1 pathway may be useful to prevent restenosis on drug eluting stents. Such compounds would be expected to reduce proliferation, migration, invasion, or other activities of cells that cause neointimal hyperplasia in a stented artery.

SUMMARY OF THE INVENTION

The present invention is directed to a method of inhibiting smooth muscle cell migration both *in vitro* and *in vivo* by administering an effective amount of a compound that inhibits signaling in the Ras-Raf-Erk-MEKK1 pathway. The present invention is further directed to the treatment of restenosis by administering such a compound via an implantable stent. Examples of such compounds include benzoquinone ansamycins.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1A-1D show the results of the migration assay using SMC. Stimulation was with 20 ng/ml human PDGF-BB plus 2.5% FBS. Paclitaxel (LC Laboratories, Woburn, MA) is included as a positive control compound for reduced SMC migration. Fig. 1 A shows the raw fluorescence readings that have only been normalized to cell number. Figs. 1B-1D have been normalized to show percent migration, where 0% represents unstimulated cells, while 100% represents fully stimulated cells.

FIGS. 2A-2D show the results of the proliferation assay using human and rat SMC and human and porcine endothelial cells (EC). EC_{50} numbers refer to the drug concentration that, after the specified incubation period, the cell number corresponds to 50% of the cell number in the untreated sample.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to a method of inhibiting smooth muscle cell migration both *in vitro* and *in vivo* by administering an effective amount of a compound that inhibits signaling in the Ras-Raf-Erk-MEKK1 pathway. The present invention is further directed to the treatment of restenosis by administering such a compound via an implantable stent. Examples of such compounds include benzoquinone ansamycins. Other types of compounds useful in the present invention are described more fully in U.S. Patent No. 4,261,989 and U.S. Patent Application Publication No. US2003/0114450 A1. In a preferred embodiment, the compound is 17-(allylamino)-17-demethoxygeldanamycin, referred to herein as 17-AAG.

The compounds for inhibiting smooth muscle cell migration or treating restenosis may be administered via an implantable device, such as a stent. Examples of implantable devices and stents that may be used in the present invention include, but are not limited to,

those described in U.S. Patent Nos. 6,709,379, 6,273,913, 5,843,172, 4,355,426, 4,101,984, 3,855,638, 5,571,187, 5,163,958 and 5,370,684; U.S. Published Patent Application Nos. US2002/0098278 and US2004/0073284; PCT International Published Patent Application No. WO 2004/043292; and European Published Patent Application No. EP 0875218.

As used herein, the term "effective amount" is intended to encompass any amount that will achieve the desired biological response.

The term "treating" in its various grammatical forms in relation to the present invention refers to preventing, curing, reversing, attenuating, alleviating, ameliorating minimizing, suppressing, or halting restenosis.

A variety of laboratory tests are available to determine whether a given compound affects the proliferation of cells in culture. For instance, the uptake of radiolabeled nucleotides into newly synthesized DNA can be measured in the presence or absence of test compounds. Alternatively, cells that have been grown in the presence or absence of test compounds can be physically counted. The preferred assay allows for the automated determination of cell number based on the color conversion of dyes by enzymes located only in the mitochondria of live cells. Using this assay, IC_{50} values (the concentration of a test compound that achieves half of its maximal antiproliferative activity), as well as the maximal antiproliferative effect can be determined for a test compound.

Several compounds that are known to impact the Ras-Raf signaling pathway in various ways were tested to determine antiproliferative activity.

Geldanamycin (InvivoGen, San Diego, CA) is a benzaquinone ansamycin antibiotic isolated from S. hydroscopicus that is known to bind to and inhibit the heat shock protein Hsp90 (Whitesell L. et al., 1994. *Proc Natl Acad Sci USA* 91(18):8324-8). One of the client proteins of Hsp90 is Raf1, so geldanamycin would be expected to inhibit Raf1. Hsp90 is a highly conserved and very abundant protein in the cytosol of both eukaryotic and prokaryotic cells. Hsp90 is a molecular chaperone critical for the folding, assembly and activity of multiple mutated and overexpressed signaling proteins that promote the growth and/or survival of tumor cells. Hsp90 plays a key role in regulating the physiology of cells exposed to environmental stress and in maintaining the malignant phenotype of tumor cells. Hsp90 client proteins play important roles in the regulation of the cell cycle, cell growth, cell survival, apoptosis, and oncogenesis. Hsp90 client proteins include mutated p53, Raf-1, Akt, ErbB2 and hypoxia-inducible factor 1a (HIF-1a) (Neckers L., 2002. *Trends Mol Med* 8(4 Suppl):S55-61). Geldanamycin binds with a high affinity into the ATP binding pocket in

Hsp90 and induces the degradation of proteins that require this chaperone for conformational maturation

17-AAG (InvivoGen, San Diego, CA) is a less toxic and more stable synthetic analog of geldanamycin (Schulte T.W. & Neckers L.M., 1998. *Cancer Chemother Pharmacol* 42(4):273-9). 17-AAG is also known to inhibit Hsp90, and thus block the Ras-Raf pathway by interfering with Raf synthesis. 17-AAG binds with a high affinity into the ATP binding pocket in Hsp90 and induces the degradation of proteins that require this chaperone for conformational maturation.

Tyrphostin AG-1295 (LC Laboratories, Woburn, MA) is one of a family of tyrosine kinase inhibitors that selectively inhibits PDGF receptor autophosphorylation, which normally signals in part via the Ras-Raf pathway (Levitzki A. & Gazit A., "Tyrosine kinase inhibition: an approach to drug development." *Science* 267: 1782-1788 (1995)). AG-1295 is also a potent inhibitor of human and porcine smooth muscle cell (SMC) proliferation *in vitro* and of rat SMC proliferation *in vivo* (Banai, S., *et al.* "PDGF-receptor tyrosine kinase blocker AG1295 selectively attenuates smooth muscle cell growth *in vitro* and reduces neointimal formation after balloon angioplasty in swine." *Circulation* 97: 1960-1969 (1998); Fishbein, I., *et al.* "Nanoparticulate delivery system of a tyrphostin for the treatment of restenosis." *J. Control Release* 65: 221-229 (2000); Karck, M., *et al.* "Inhibition of aortic allograft vasculopathy by local delivery of platelet-derived growth factor receptor tyrosine-kinase blocker AG-1295." *Transplantation* 74: 1335-1341 (2002)).

Tyrphostin AG-1478 (LC Laboratories, Woburn, MA) is one of a family of tyrosine kinase inhibitors that selectively inhibits EGF receptor kinase, which signals in part via the Ras-Raf pathway (Osherov, N. and Levitsky, A. *Eur. J. Biochem.* 225: 1047-1053 (1994); Levitsky, A. and Gazit, A. *Science* 267: 1782-1788 (1995)).

PD-98059 or 2'-amino-3'-methoxyflavone (LC Laboratories, Woburn, MA) is a selective inhibitor of mitogen activated protein kinase kinase (MEK-1 and MEK-2); i.e., inhibits phosphorylation of MAP kinase by MAP kinase kinase (Dudley, D.T. *et al. Proc. Natl. Acad. Sci. USA* 92: 7686-7689 (1995); Pang, L. *et al. J. Biol. Chem.* 270: 13585-13588 (1995). Waters, S.B. *et al. J. Biol. Chem.* 270: 20883-20886 (1995); Langlois, W.J. *et al. J. Biol. Chem.* 270: 25320-25323 (1995); Alessi, D.R. *et al. J. Biol. Chem.* 270: 27489-27494 (1995)). This is an intermediate in the signaling pathway downstream of Ras/Raf.

UO-126 or 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)-butadiene (LC Laboratories, Woburn, MA) is a selective inhibitor of MEK-1 and MEK-2, which are downstream effectors in the Ras-Raf pathway (Favata, M.F. *et al.* "Identification of a novel

inhibitor of mitogen-activated protein kinase kinase." *J. Biol. Chem.* 273: 18623-18632 (1998); DeSilva, D.R. *et al.* "Inhibition of mitogen-activated protein kinase kinase blocks T cell proliferation but does not induce or prevent anergy." *J. Immunol.* 160: 4175-4181 (1998); Scherle, P.A. *et al.* "Inhibition of MAP kinase kinase prevents cytokine and prostaglandin E2 production in lipopolysaccharide-stimulated monocytes." *J. Immunol.* 161: 5681-5686 (1998)).

Raf1 Kinase Inhibitor I or 5-iodo-3-[(3,5-dibromo-4-hydroxyphenyl)methylene]-2-indolinone (Calbiochem, San Diego, CA) is a potent inhibitor of cRAF1 kinase, which is the activity of Raf that is responsible for phosphorylation of MEK. This inhibitor is highly selective for Raf kinase versus other kinases such as Cdk1, Cdk2, c-Src, ERK2, MEK, p38, Tie2, VEGFR2, and c-Fms (Lackey, K., et al. 2000. *Bioorg. Med. Chem. Lett.* 10, 223).

MCP-110 (Morphochem AG, Munich, Germany) is an inhibitor of Ras-Raf interaction, and has been shown to revert Ras-dependent phenotypes in cancer cells (Kato-Stankiewicz et al. (2002) PNAS 99:14398-14402).

EXAMPLE 1:

Migration Assay

This assay primarily measures chemotaxis, because it quantifies the number of cells that move through a porous membrane toward a chemoattractant (e.g., serum or growth factor) in a given period of time. Nevertheless, there may also be a component of chemokinesis in the assay, due to the diffusion of chemoattractants across the membrane, potentially resulting in the general activation of the cells to move randomly.

Primary cells and cell culture media are obtained from Clonetics (Walkersville, MD) and are grown at 37°C with 5% CO_2. Primary human, porcine, or rat coronary artery smooth muscle cells (CASMC) are used at passage number ≤ 6. They are grown in smooth muscle cell basal medium (modified MCDB 131), with the addition of: 5% fetal bovine serum (FBS); 0.5 μg/ml human epidermal growth factor (hEGF); 5 mg/ml insulin; 1.0 μg/ml human fibroblast growth factor; 50 mg/ml gentamycin; and 50 μg/ml amphoteracin B.

Primary human, porcine, or rat coronary vascular endothelial cells (CVEC) are used at passage number ≤ 10. They are grown in endothelial cell basal medium (modified MCDB 131), with the addition of: 5% FBS; 10 μg/ml hEGF; 1.0 mg/ml hydrocortisone; 3 mg/ml bovine brain extract; 50 mg/ml gentamycin; and 50 μg/ml amphoteracin B.

Cells are removed from flasks by brief exposure to trypsin-EDTA (Invitrogen), followed by inactivation with trypsin neutralizing solution (Clonetics), centrifugation for 5 minutes at 2,000 rpm, and resuspension in basal medium containing 0.4% FBS at a concentration of 2.0×10^5 cells per milliliter. 0.25 ml cell suspension is pipetted into the upper chambers of BD Falcon FluoroBlok™ 24 well insert plates (modified Boyden chambers) (BD Biosciences, Bilerica, MA), containing fibronectin-coated filters with 8 μm pores. Each lower chamber contains basal medium with the addition of chemoattractants such as serum or growth factors. For HCASMC, FBS and/or human platelet derived growth factor BB (hPDGF-BB) is used. For HCVEC, FBS and/or human vascular endothelial growth factor (hVEGF) is used.

After cells are added to the top chamber, along with varying concentrations of drugs that are intended to inhibit migration, the bottom chamber is filled with 0.75 ml of basal medium containing chemoattractants and identical drug concentrations as the upper chamber. The plates are then incubated for either 6 hours or 22 hours at 37°C.

Remaining treated cells are analyzed in the WST-1 proliferation assay (50 μl cells / well in quadruplicate) to determine relative cell number in each sample of the assay. This reading is used to normalize the final results to cell number in each test sample.

At the end of the incubation period, liquid in the top chamber of each well is aspirated, and the top half of the plate (containing the 24 upper chambers to which the permeable filters are fused) is lifted off, and excess liquid is shaken into a sink. The top half of the plate is then placed into a fresh 24-well plate, each well of which contains 0.75 ml Calcein AM solution (4 μg/ml, Molecular Probes, Eugene, OR). The complete assembly is then incubated at 37°C for 60 minutes, during which time the Calcein AM stains the cells that remained attached to the filter. The stained plate is then placed in a Victor II plate reader (PerkinElmer, Boston, MA) that is programmed to read from the bottom, with excitation at 485 nm, emission at 535 nm, and a 0.1 second read time. Since the filter through which the cells have migrated contains a dark, opaque pigment, the excitation or emission light does not penetrate the filter. Thus, only cells that have migrated through to the underside of the filter are detected by the fluorescence detector.

Raw data is expressed as arbitrary fluorescence units, which is subsequently normalized to cell number and analyzed using Prism v 3.02 (Graphpad Software). Data is expressed as either direct comparisons among unstimulated, stimulated, and stimulated cells with drug or, alternatively, percent migration (with unstimulated being 0% and stimulated being 100%).

This migration assay was conducted using AG-1295, 17-AAG, UO-126, PD-98059 and paclitaxel. Paclitaxel was included as a positive control compound for reduced SMC migration. Figure 1A shows the raw fluorescence readings that have only been normalized to cell number. The compounds were tested in human SMC using 20 ng/ml of human PDGF-BB plus 2.5% FBS as stimulus. Figures 1B-1D show results normalized to show percent migration, where 0% represents unstimulated cells and 100% represents fully stimulated cells. Figure 1B shows the results of testing the compounds in human SMC using 20 ng/ml of human PDGF-BB plus 2.5% FCS as stimulus. Figure 1C shows the results of testing the compounds in porcine SMC using 20 ng/ml of human PDGF-BB plus 2.5% FCS as stimulus. Figure 1D shows the results of testing the compounds in rat SMC using 20 ng/ml of human PDGF-BB plus 2.5% FCS as stimulus.

Table 1 shows the results of migration assays conducted using various compounds described herein.

Table 1: Results of Migration Assays

Compound	Porcine SMC	Rat SMC	Human SMC
AG-1478	75% 10μM	80% 10μM	80% 10μM
AG-1295	10% 10μM	100% 10μM	60% 10μM
17-AAG	-7% 5μM	-30% 5μM	-6% 5μM
UO-126	60% 5μM	70% 5μM	90% 5μM
XC-221	100% 50μM	80% 40μM	40% 50μM
Paclitaxel	25% 5μM	-15% 5μM	5% 5μM
PD-98059	90% 5μM	100% 5μM	100% 5μM

EXAMPLE 2:

Proliferation Assay

This assay measures the number of live cells in a tissue culture dish or well. It does so by monitoring the color change of the tetrazolium salt, WST-1 that is modified by a mitochondrial enzyme involved in respiration. This enzyme is only active in living cells. The readout from this assay provides a linear correlation with live cell number in the conditions that are used here.

Primary cells and cell culture media are obtained from Clonetics (Walkersville, MD) and are grown at 37°C in a humidified incubator containing 5% CO_2. Primary human coronary artery smooth muscle cells (HCASMC) or other human vascular SMC are used at

passage number ≤ 10. They are grown in smooth muscle cell basal medium (modified MCDB 131), with the addition of: 5% fetal bovine serum (FBS); 0.5 μg/ml human epidermal growth factor (hEGF); 5 mg/ml insulin; 1.0 μg/ml human fibroblast growth factor; 50 mg/ml gentamycin; and 50 μg/ml amphoteracin B. In some assays, cells are induced to proliferate with other mitogens, such as thrombin or PDGF, combined with various levels of FBS. Alternatively, higher amounts of FBS, up to 10% or 20% can be used.

Primary human coronary vascular endothelial cells (HCVEC) or other human endothelial cells (EC) are used at passage number ≤ 10. They are grown in endothelial cell basal medium (modified MCDB 131), with the addition of: 5% FBS; 10 μg/ml hEGF; 1.0 mg/ml hydrocortisone; 3 mg/ml bovine brain extract; 50 mg/ml gentamycin; and 50 μg/ml amphoteracin B. In some assays, cells are induced to proliferate with other mitogens, such as thrombin or PDGF, in which case 5% FBS is replaced by 0.1% FBS.

Cells are removed from flasks by brief exposure to trypsin-EDTA (Invitrogen), followed by inactivation in complete medium, centrifugation for 5' at 2,000 rpm, and resuspension in test medium. Cells are counted using a hemocytometer and plated into 96-well tissue culture plates at $5x10^3$ cells/well in 50 μl test medium.

Test compounds are dissolved either in DMSO or PBS, such that the final concentration of DMSO in the assay is 0.5% or less, typically 0.2% or 0.4%. Compounds are prepared at twice the final assay concentration in test medium, and 50 μl is added to each well. The plates are then incubated for 2 – 5 days at 37°C.

At the end of the incubation period, 10 μl WST-1 reagent (Roche Molecular Biochemicals, Indianapolis, IN) is added to each well, followed by incubation at 37°C for 90 minutes. During this time, the color change in the WST-1 reagent correlates with the number of live cells in each well. At the end of the incubation period, plates containing live cells can be analyzed immediately, or 15 μl of 10% sodium dodecyl sulfate (SDS) can be added to each well, thus lysing the cells and preserving the assay for later analysis. Plates are analyzed (0.1 second/well) for absorbance at 450 nm in a Victor II plate reader (PerkinElmer, Boston, MA).

Data is expressed as arbitrary absorbance units (correlating with the number of live cells) and analyzed using Prism v 3.02 (Graphpad Software). Controls are wells that contain no cells (this absorbance reading is subtracted from all test wells) and wells in which cells do not proliferate due to presence of low serum (0.1% FBS) or added mitogens – this number

represents live cells that have not proliferated during the course of the assay, and can demonstrate the effect of compounds that are cytotoxic in addition to being antiproliferative.

This proliferation assay was conducted using the compounds UO-126, AG1295, 17-AAG and AG-1478. The results are shown in Figures 2A-2D. EC_{50} numbers refer to the calculated molar drug concentration at which the final cell number corresponds to 50% of the cell number in the untreated sample.

As indicated by the results of these assays, treatment of coronary vascular SMC or EC by different inhibitors of the Ras-Raf pathway results in varying degrees of cell proliferation and/or cell migration inhibition. By extension, one or more of these or other similar inhibitors could inhibit proliferation, migration, invasion or other cellular activities that lead to restenosis in stented blood vessels. Since the capacity of *in vitro* tests to predict activity in animal models of restenosis or in humans varies widely due to potency, selectivity, and pharmacodynamics, any of the inhibitors of the Ras-Raf pathway could prove to effectively inhibit restenosis in a clinical setting.

Based on these results, it is clear that 17-AAG, a benzoquinoid ansamycin, is more effective in cell migration inhibition than paclitaxel and is also effective in cell proliferation inhibition. From these *in vitro* results, it is concluded that 17-AAG and other benzoquinoid ansamycin compounds are effective *in vivo* in the inhibition of cell migration and/or cell proliferation and the treatment of restenosis. A more effective way of administering the compounds described herein is to apply the compound to an implantable stent. Implantable stents can be made according to methods known in the art, such as those set forth in U.S. Patent Application Publication No. US2002/0127263 A1.

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the meaning of the invention described. The scope of the invention includes the subject matter of the claims that follow. All patents, patent applications, and publications cited herein are hereby incorporated by reference.

What is claimed is:

1. A method of inhibiting smooth muscle cell migration comprising the administration of an effective amount of a compound that inhibits signaling in the Ras-Raf-Erk-MEKK1 pathway, wherein the compound is incorporated in an implantable device.
2. The method of claim 1 wherein the compound is a benzoquinone ansamycin.
3. The method of claim 2 wherein the benzoquinone ansamycin is 17-(allylamino)-17-demethoxygeldanamycin (17-AAG).
4. The method of claim 1 wherein the implantable device is a stent.
5. A method of treating restenosis comprising the administration of an effective amount of a compound that inhibits signaling in the Ras-Raf-Erk-MEKK1 pathway, wherein the compound is incorporated in an implantable device.
6. The method of claim 5 wherein the compound is a benzoquinone ansamycin.
7. The method of claim 6 wherein the benzoquinone ansamycin is 17-(allylamino)-17-demethoxygeldanamycin (17-AAG).
8. The method of claim 5 wherein the implantable device is a stent.
9. A method of treating restenosis comprising the administration of an effective amount of 17-(allylamino)-17-demethoxygeldanamycin (17-AAG) via an implantable stent.

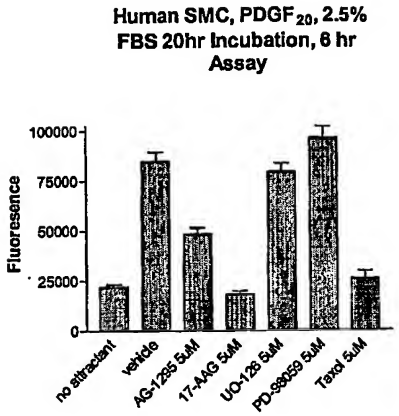


FIG. 1A

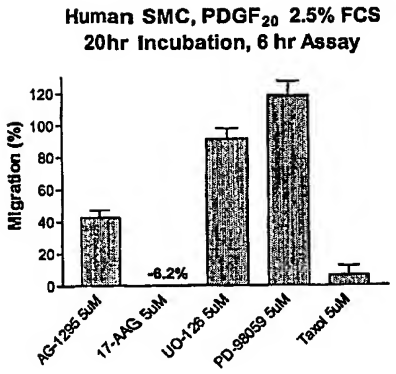


FIG. 1B

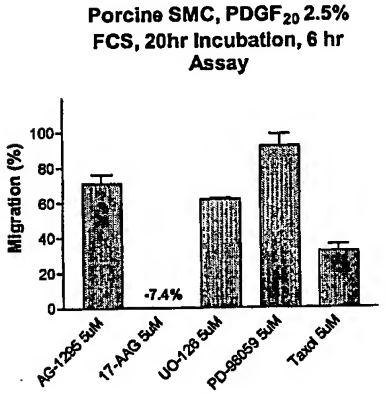


FIG. 1C

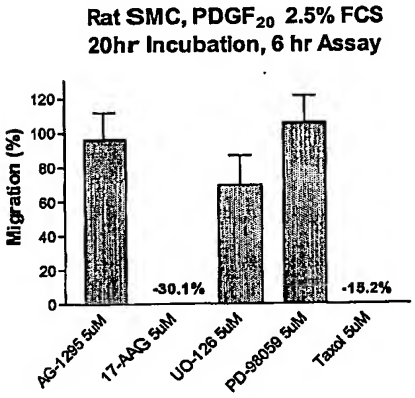
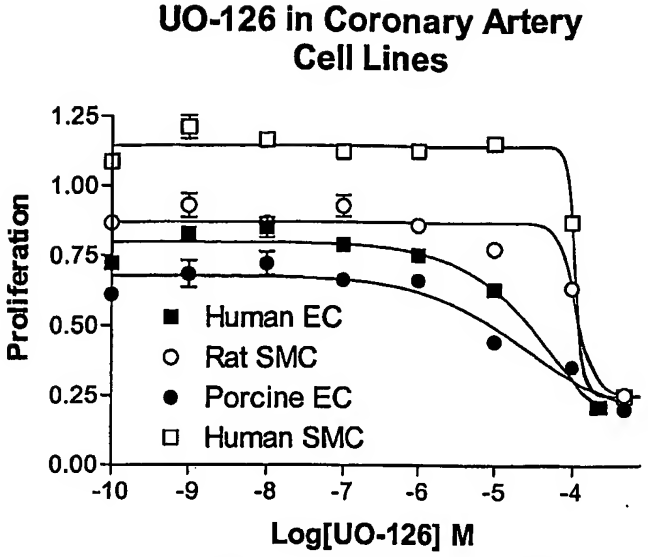
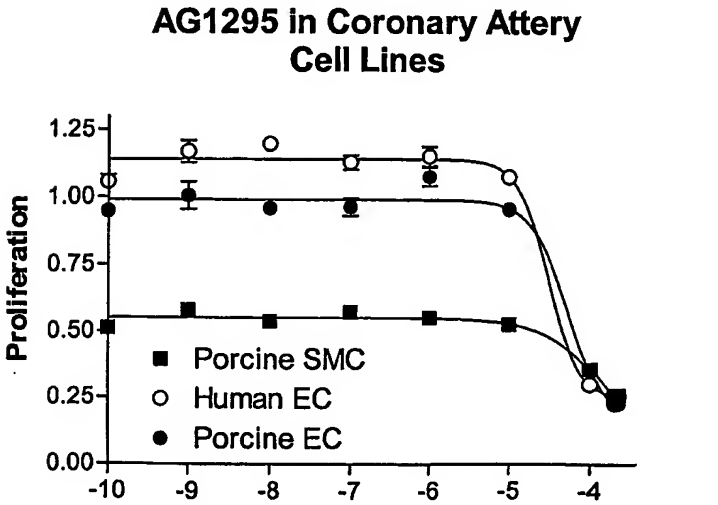


FIG. 1D



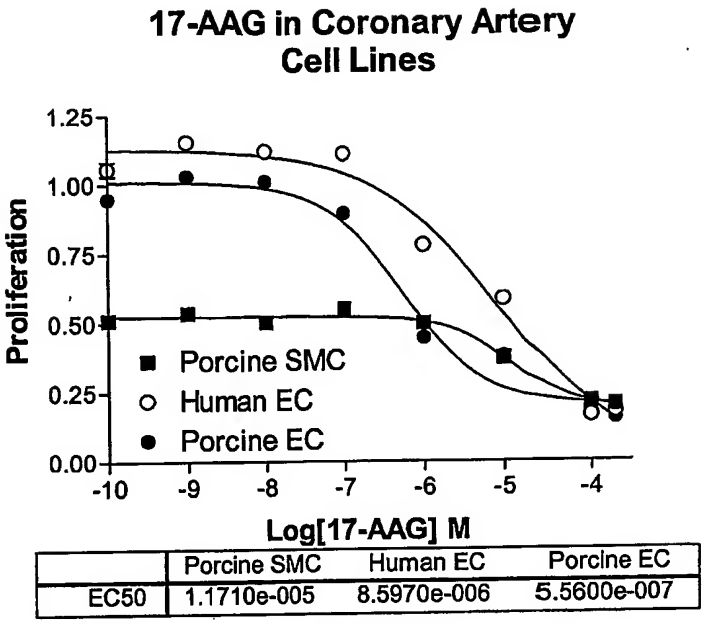
	Human EC	Rat SMC	Porcine EC	Human SMC
EC50	7.0020e-005	0.0001128	2.8510e-005	0.0001101

FIG. 2A



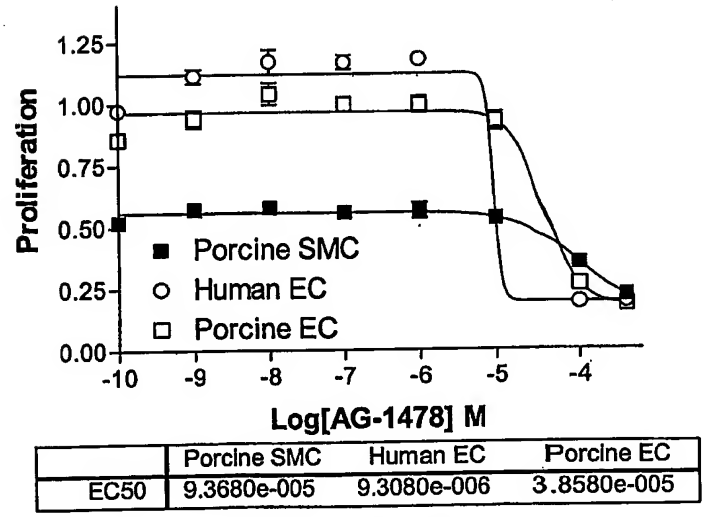
	Porcine SMC	Human EC	Porcine EC
EC50	0.0001503	3.0700e-005	5.2440e-005

FIG. 2B



	Porcine SMC	Human EC	Porcine EC
EC50	1.1710e-005	8.5970e-006	5.5600e-007

FIG. 2C



	Porcine SMC	Human EC	Porcine EC
EC50	9.3680e-005	9.3080e-006	3.8580e-005

FIG. 2D